REMARKS

Applicants respectfully request reconsideration of this application in view of the foregoing amendments and the following remarks.

A. Introductory Remarks

Upon entry of the foregoing amendments, claims 15-25 will remain pending in the application. Claims 1-14 are presently being canceled without prejudice or disclaimer, solely because they are withdrawn from consideration. No claims are presently being added.

B. The Claims Comply with the Enablement Requirement of 35 U.S.C. § 112

Claims 15-19 and 21-25 were rejected for allegedly failing to meet the enablement requirement of 35 U.S.C. § 112, first paragraph. The Office acknowledged that the specification is "enabling for methods of treating malignant skin lesions with 5-aminolevulinic acid (5-ALA)," but stated that it does not enable the use of "any agent which is not itself a photosensitizer but which induces accumulation of protoporphyrin IX in a cellular target." According to the Office, "the scope of applicable agents that can provide the claimed function is unknown" and "narrowing the range of candidates in order to find a suitable compound" is too difficult. Applicants respectfully traverse the rejection.

The scope of compounds useful in the invention was known to those skilled in the art, who would have understood that "an agent which is not a photosensitizer but induces the synthesis of protoporphyrin IX in vivo" is a compound capable of participating, either directly or after an *in vivo* conversion, in the heme biosynthetic pathway and inducing synthesis of protoporphyrin IX. For an illustration of the heme biosynthetic pathway, Applicants refer the Examiner to McGilvery et al., Biochemistry: A Fundamental Approach 632-635 (2d ed. 1979) (Exhibit 1). This textbook shows the heme biosynthetic pathway and illustrates the role of 5-aminolevulinate. Compounds useful in the invention include those compounds that are involved in the biosynthetic pathway of heme as shown in McGilvery.

Additionally, the present specification discloses at paragraph 0030 that "the usual rate-limiting step in the [heme biosynthetic] process, the synthesis of 5-aminolevulinic acid, can

be bypassed by the provision of exogenous ALA, porphobilinogen or other precursor of PpIX." Those ordinarily skilled in the art would understand an "other precursor" of protoporphyrin IX to include prodrugs of compounds in the heme biosynthetic pathway. Such an "other precursor" would include, for example, an ester of 5-aminolevulinic acid. To support this position, Applicants offer, De Matties et al., "Brain 5-aminolaevulinate synthase," Biochemical Journal, vol. 196, 811-817 (1981) (Exhibit 2). De Matties et al. disclose that ". . . the methyl ester [of 5-aminolevulinic acid] reflects passive diffusion of the unchanged methyl ester across the blood/brain barrier, followed by hydrolysis to the free amino acid within the brain and subsequent conversion of 5-aminolaevulinate into haem." This reflects knowledge at the time of the invention that an agent such as an ester of 5-aminolevulinic acid could be administered to a patient to achieve conversion into heme and, accordingly, synthesis of protoporphyrin IX (a precursor to heme).

Also, Applicants submit Srivastava et al., "Regulation of 5-Aminolevulinate Synthase mRNA in Different Rat Tissue," Journal of Biological Chemistry, vol. 263, 5202-5209 (1988) (Exhibit 3) to further support the knowledge at the time of the invention that an ester of 5-aminolevulinic acid, such as the methyl ester, was known to enter the heme biosynthetic pathway via 5-aminolevulinic acid and therefore is active in inducing synthesis of protoporphyrin IX (a precursor to heme). Srivastava et al. disclose that "[a]dministration of hemin to rats reduced the basal level of this mRNA [a specific 5-aminolevulinate synthase] only in liver, but the heme precursor, 5-aminolevulinate (or its methyl ester) repressed the basal levels in liver, kidney, heart, testis and brain."

Thus, at the time of the invention, the phrase "agent which is not a photosensitizer but induces the synthesis of protoporphyrin IX in vivo" included substances such as 5-aminolevulinic acid as described in the present specification, the shown and listed precursors of PpIX as described by McGilvery in Biochemistry and esters of 5-aminolevulinic acid, such as the methyl ester of 5-aminolevulinic acid as described by Srivastava et al. and De Matties et al.

In view of the foregoing amendments and comments, Applicants respectfully request reconsideration and withdrawal of the enablement rejection.

C. Double Patenting

Claims 15-25 were rejected on the basis of obviousness-type double patenting over claims 1-2 of U.S. Patent No. 5,211,938 and claims 1-7 of U.S. Patent No. 5,069,262. Once the claims in this application are otherwise deemed allowable, Applicants intend to file a terminal disclaimer that will obviate this rejection.

Claims 15-25 were provisionally rejected on the basis of obviousness-type double patenting over claims 1-10 of copending U.S. Patent Application No. 10/663,992, claims 1-20 of copending U.S. Patent Application No. 10/605,826, claims 1-19 of copending U.S. Patent Application No. 09/928,505 and claims 1-15 of copending U.S. Patent Application No. 09/816,329. Because the rejection is merely provisional at this time, Applicants defer any action until an actual rejection is made.

D. The Claims Are Patentable over the Cited Art

Claims 15-19 and 21-25 were rejected under 35 U.S.C. § 103(a) as allegedly being obvious over U.S. Patent 5,093,349 ("Pandey"). According to the Office, Pandey teaches using "dimmers of deuteroporphyrins or hydrophobic esters thereof" in photodynamic therapy of skin tumors. Applicants traverse the rejection.

Pandey neither teaches nor suggests the claimed method for treating malignant skin lesions. The claimed method requires administering "an agent which is not a photosensitizer but induces the synthesis of protoporphyrin IX in vivo." The compounds described in Pandey do not meet either of those requirements. Indeed, Pandey explains at column 10, lines 56-65, that its described compounds "are [not] entered into any biological event" and "are not consumed or altered in exerting their biological effects." Because the compounds described by Pandey are not altered, they must already be photosensitizers at the time of administration. Likewise, because the compounds described by Pandey do not enter into any biological event and are not altered, they must not induce the synthesis of protoporphyrin IX in vivo.

Pandey does not suggest using any compounds beyond those that it describes, and the Office did not identify any suggestion or motivation beyond Pandey to do so. Accordingly,

the claimed invention is patentable over Pandey, and Applicants request withdrawal of the obviousness rejection.

E. Concluding Remarks

This application is now in condition for allowance, and Applicants respectfully request favorable reconsideration of it.

If the Examiner believes that an interview would further advance prosecution, he or she is invited to contact the undersigned attorney by telephone.

The Commissioner is hereby authorized to charge any additional fees that may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicants hereby petition for such extension under 37 C.F.R. §1.136 and authorize payment of any extension fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 10 November 2005

By _____

FOLEY & LARDNER LLP
Customer Number: 22428
Telephone: (202) 672-5475
Facsimile: (202) 672-5399

Beth A. Burrous
Attorney for Applicant
Registration No. 35,087

SECOND EDITION

BIOCHEMISTRY

A Functional Approach

R. W. McGILVERY, Ph.D.

Professor of Biochemistry,
University of Virginia School of Medicine

in collaboration with

GERALD GOLDSTEIN, M.D.

Professor of Internal Medicine,
University of Virginia School of Medicine

1979

W. B. SAUNDERS COMPANY

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	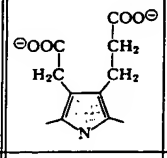	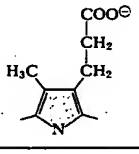	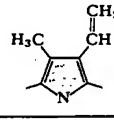	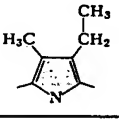	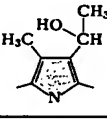	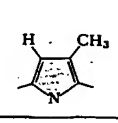
uroporphyrin	4	—	—	—	—	—
coproporphyrin	—	4	—	—	—	—
protoporphyrin	—	2	2	—	—	—
etioporphyrin	—	—	—	4	—	—
hematoporphyrin	—	2	—	—	2	—
mesoporphyrin	—	2	—	2	—	—
deuteroporphyrin	—	2	—	—	—	2

FIGURE 33–1 **Kinds of porphyrins. The nature of the constituent pyrroles as listed at the top defines the porphyrin, and the number of the various pyrroles in different kinds of porphyrins is given. It is not hard to visualize sequences of decarboxylations, oxidations, hydrations, and reductions by which all of these could be formed from the parent uroporphyrins listed first.**

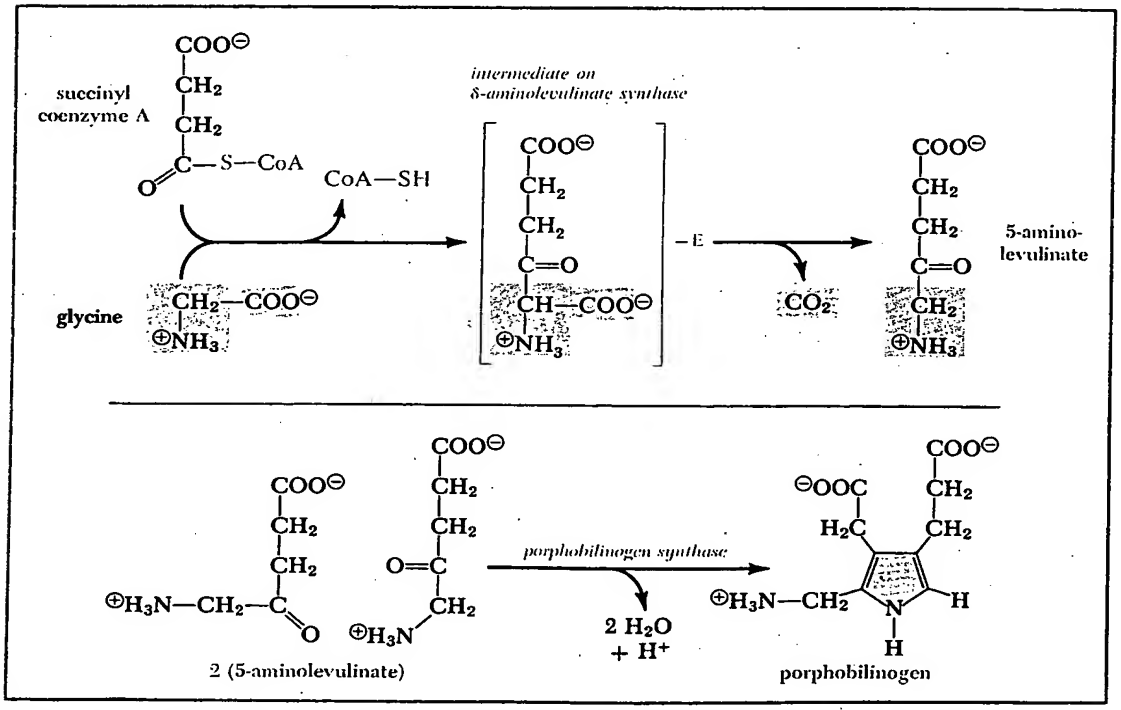

FIGURE 33–2 **Porphyrin synthesis begins with two successive condensations by which a pyrrole ring is generated from two molecules each of succinyl coenzyme A and glycine.**

Each of these four uroporphyrins is designated by a Roman numeral. (There are only four uroporphyrins because any other reversal of pyrrole groups beyond those shown is superimposable on one of the four by turning the ring over.)

Now, if two of the groups in uroporphyrin are changed into a third kind of group, which is the circumstance seen in protoporphyrins, then there are 15 possible combinations. Hans Fischer* wrote down the 15 possibilities, and showed that the porphyrin in hemoglobin had the same arrangement as the ninth he had tabulated. Hence, the porphyrin in heme is designated as protoporphyrin IX.

All natural porphyrins are derived from uroporphyrin I, in which there is a regular alternating sequence of groups, as might be expected if the pyrroles are combined head-to-tail, and from uroporphyrin III, which represents a reversal — an isomerization — of one of the pyrrole groups.

PORPHYRIN SYNTHESIS

The complex porphyrin molecule is made from two simple precursors, **succinyl coenzyme A** and **glycine** (Fig. 33–2). The initial reaction is a condensation of these compounds within mitochondria, where they are readily available, to form **5-aminolevulinate.** This is the rate-controlling step in porphyrin biosynthesis. The 5-aminolevulinate passes into the cytosol for the next step.

The reaction involves an intermediate condensation of glycine with pyridoxal phosphate. The mechanism is not shown; one of the H atoms on C-2 of glycine leaves after condensation; the resultant carbanion then unites with the electropositive carbonyl carbon of succinyl coenzyme A.

Two molecules of 5-aminolevulinate condense to form **porphobilinogen.** This is the parent pyrrole compound, and four molecules of it are combined to make **uroporphyrinogen III** (Fig. 33–3).

*Hans Fischer (1881–1945): German biochemist and Nobel Laureate. Not to be confused with Emil Fischer (1852–1919), also a German biochemist and Nobel Laureate, discoverer of much of the fundamental knowledge of the chemistry of proteins, carbohydrates, and nucleic acids; nor with Emil Fischer's late son, H. O. L. Fischer, a carbohydrate chemist of distinction at Toronto and Berkeley; nor with E. H. Fischer, very much alive at Seattle, and not bad as a biochemist, either. (This list is by no means exhaustive.)

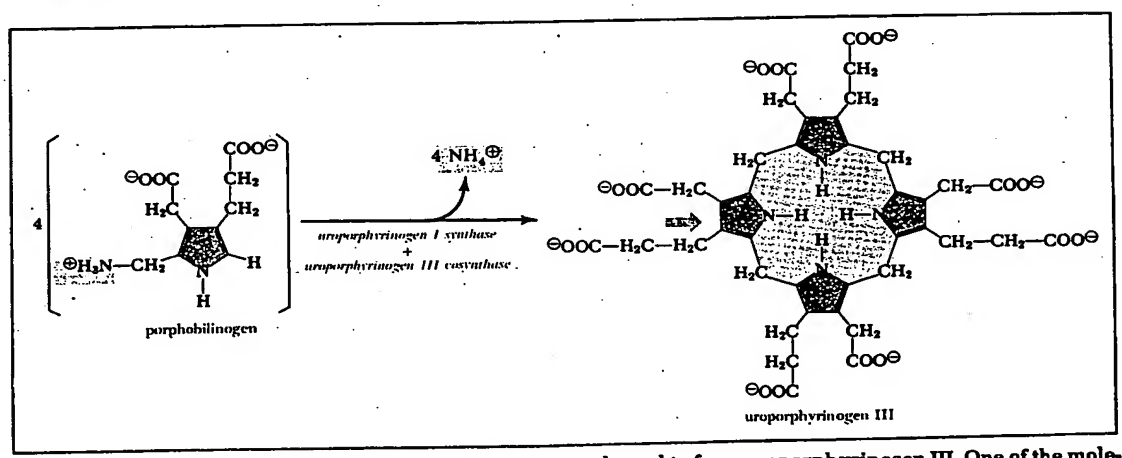

FIGURE 33–3 Four molecules of porphobilinogen are condensed to form uroporphyrinogen III. One of the molecules *(arrow)* condenses head-to-head in the presence of a cosynthase; the others condense head-to-tail.

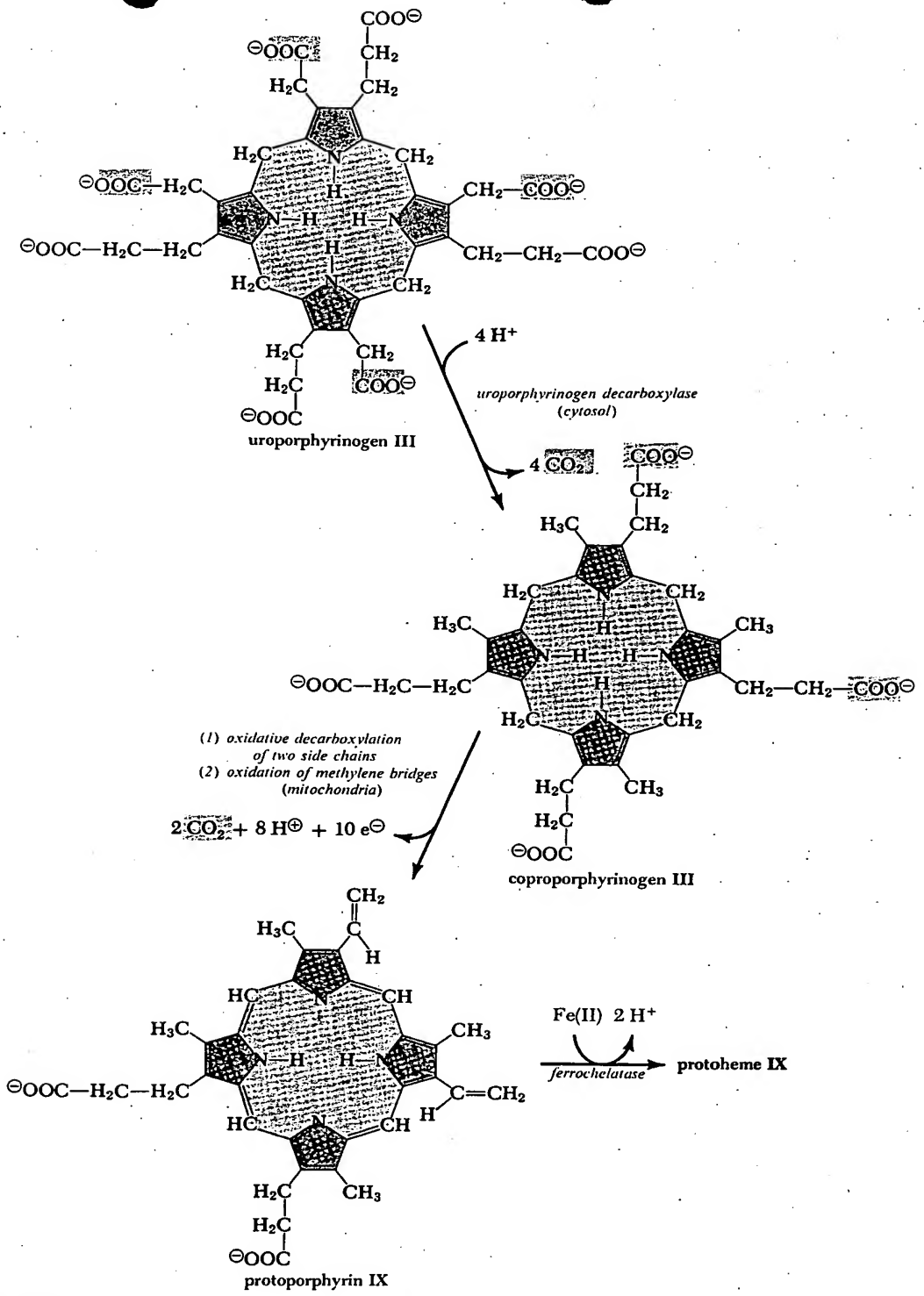

FIGURE 33–4 The conversion of uroporphyrinogen III to protoporphyrin IX and then to protoheme IX.

(The **porphyrinogens** are porphyrins in which the bridge atoms between pyrrole rings are in the reduced, or methylene, state, whereas these atoms are in the methylidyne state in porphyrins.) Two proteins are involved in this condensation. Uroporphyrinogen I synthase by itself would catalyze a simple head-to-tail condensation of porphobilinogen units, forming uroporphyrinogen I. The second protein, a uroporphyrinogen III cosynthase, has no apparent catalytic activity, but it somehow combines with the synthase so as to alter its specificity, causing one of the porphobilinogen molecules to condense head-to-head, creating a type III porphyrin. The mechanism is unknown.

The remaining steps (Figs. 33–4) involve decarboxylation of the aceto side chains to form methyl groups (coproporphyrinogen III), oxidative decarboxylation of two of the propiono side chains to form vinyl groups (protoporphyrinogen IX), and the oxidation of the methylene bridges to methylidyne bridges (protoporphyrin IX). The latter two steps are catalyzed by mitochondria, but it is not known where the enzymes are localized within the organelle. However, the final step of

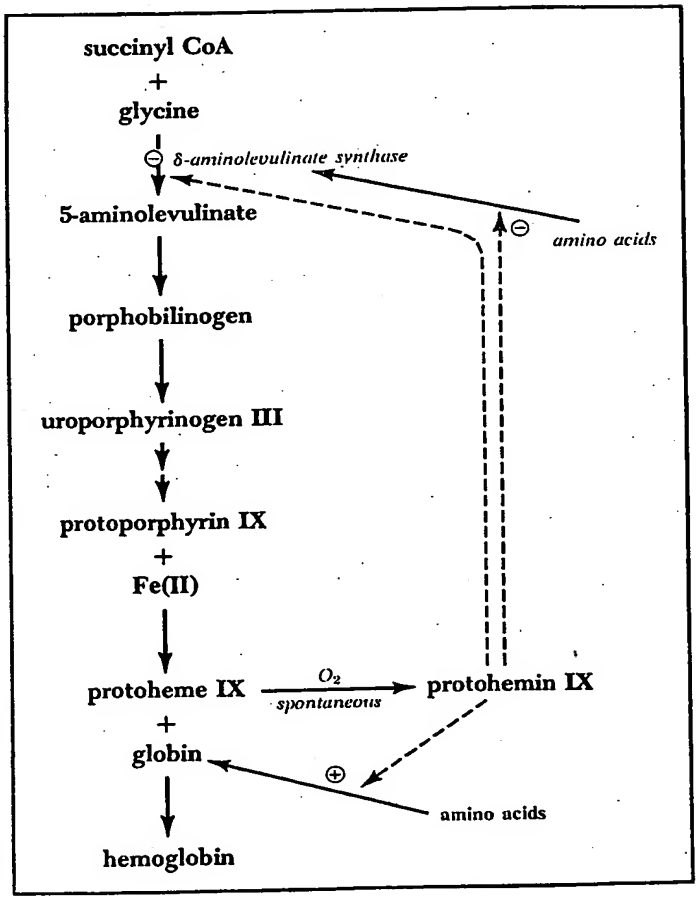

FIGURE 33–5

Regulation of hemoglobin synthesis by protohemin IX. Hemin forms when the supply of heme exceeds the supply of globin. The hemin suppresses formation of additional protoporphyrin, probably by direct inhibition of aminolevulinate synthase and also by repression of the enzyme's synthesis. The hemin also promotes synthesis of globin polypeptides.

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Brain 5-aminolaevulinate synthase

Developmental aspects and evidence for regulatory role

Francesco DE MATTEIS,* Per ZETTERLUND and Lennart WETTERBERG
Department of Psychiatry, Karolinska Institute, St. Göran's Hospital, Box 12500, S-112 81 Stockholm, Sweden

(*Received 5 January 1981/Accepted 13 March 1981*)

1. Brain 5-aminolaevulinate synthase showed a peak of increased activity in the first few weeks of life, which preceded and accompanied the development of brain cytochromes. 2. In the brain of the adult rat the activity of the enzyme was only 20% of that in the liver (on a per g wet wt. basis), but it was still probably sufficient to maintain the turnover of brain cytochromes. 3. The brain synthase activity could be decreased by treatment of rats with cycloheximide or with large doses of 5-aminolaevulinate, especially when this precursor was given as the methyl ester. 4. Injected haematin and $CoCl_2$ markedly inhibited the synthase activity in the liver but failed to affect the brain enzyme; neither was taken up by the brain *in vivo*. 5. It is concluded that the brain can itself produce the haem required for the synthesis and turnover of its own haemoproteins and that 5-aminolaevulinate synthase may regulate the pathway in brain as in other tissues. 6. The relevance of the present findings to the pathogenesis of the neurological symptoms of acute porphyria and to the beneficial effect of exogenous haematin in porphyric patients is briefly considered.

Acute intermittent porphyria is an inborn error of metabolism characterized by a partial block in liver haem biosynthesis and by increased urinary excretion of the intermediates of the pathway, 5-aminolaevulinate and porphobilinogen. During the phases of exacerbation of the disease, or acute attacks, the excretion of 5-aminolaevulinate and porphobilinogen becomes even greater and a number of symptoms may appear, all attributable to impairment of nervous function. Thus, although the liver appears to be the main site where the intermediates of the pathway are produced in excess, the clinical symptoms pertain almost exclusively to the nervous system.

There are two main hypotheses for the pathogenesis of the neurological symptoms of the acute attack (Shanley *et al.*, 1977; Meyer & Schmid, 1974). (1) The excessive amounts of porphyrin precursors made in the liver may lead to a build up within the nervous system of a toxic principle responsible for the neurological symptoms. (2) The partial block of haem biosynthesis due to the genetic defect may be expressed in the nervous tissue (as well as in the liver) and lead there to a decreased supply of haem, which may then in turn cause impaired metabolism and loss of function of the neurons. Large doses of exogenous haem are said to be beneficial when given to a porphyric patient during an acute attack (Watson *et al.*, 1973; Jeelani Dhar *et al.*, 1975; Watson, 1975; Peterson *et al.*, 1976). This beneficial effect could be explained by either of the two hypothetical mechanisms considered above: exogenous haem is known to inhibit formation of the intermediates of the pathway in the liver (by lowering the activity of hepatic 5-aminolaevulinate synthase; Bonkowsky *et al.*, 1971), and could in this way prevent the formation of the postulated neurotoxic principle; also, if haem were taken up by the brain in sufficient amounts, it could conceivably rectify a state of haem deficiency in the nervous system.

The work described in the present paper was stimulated by our interest in the biochemical mechanism of the nervous manifestations of porphyria and was aimed at answering two basic questions. (1) Is the brain capable of producing haem for its own needs, even under conditions where haem requirements are increased, or does it depend

* Present address: Biochemical Pharmacology Section, MRC Toxicology Unit, MRC Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

0306-3283/81/060811-07$01.50/1

on other organs and tissues (for example on the liver; Bissell *et al.*, 1979; Maines, 1980) for its haem requirement? (2) Can exogenously administered haem be taken up by the brain *in vivo*?

Materials and methods

Animals

Male Sprague-Dawley rats were allowed food and water *ad lib.* and were adjusted to a 12h light/12h darkness cycle for at least 1 week before killing. With the exception of rats given cycloheximide, $CoCl_2$, haematin or 5-aminolaevulinate *in vivo*, where individual rats were studied, the livers and brains from two animals were pooled for each observation. The effect of cycloheximide and $CoCl_2$ was also studied in male Porton rats with very similar results. Animals were killed by decapitation (between 11:00h and 13:00h) and their brain and liver were obtained within 30s and transferred into an ice-cold homogenizing fluid containing sucrose (0.25M), Tris/HCl buffer, pH7.2 (10mM) and EDTA (1mM). The whole brain was taken for homogenization, including the cerebellum.

Methaemalbumin was prepared (Tenhunen *et al.*, 1968), using bovine serum albumin; [^{14}C]haemin (sp. radioactivity 0.1Ci/mol) was prepared biosynthetically from 5-amino[4-^{14}C]laevulinate. 5-Aminolaevulinic acid and its methyl ester were injected intravenously dissolved in a propylene glycol/saline (0.9% NaCl) mixture (1:2, v/v) and $CoCl_2$ and cycloheximide dissolved in physiological saline. Doses and time of killing are given in the Results section.

Studies in vitro

5-Aminolaevulinate synthase (EC 2.3.1.37) was assayed by a slight modification of the radiochemical method described by Condie & Tephly (1978). The incubation mixture contained in a total volume of 2ml the following components with final concentrations, or total amounts, in parentheses: liver or brain homogenate (equivalent to 100mg wet wt. of tissue), Tris/HCl buffer (75mM), glycine (100mM), EDTA (7.5mM), pyridoxal 5′-phosphate (0.4mM), ATP (1.36mM) and [2,3-^{14}C]succinate (sp. radioactivity 2.73Ci/mol; 0.37mM). The pH of the complete mixture was 7.5 at 20°C. Incubation was for 1h at 38°C in the presence of air but without shaking and zero-time samples and incubated blanks (containing no enzyme) were routinely run. Under these conditions conversion of [2,3-^{14}C]succinate into 5-aminolaevulinate was linear with time of incubation (up to at least 1h) and with amount of tissue taken (up to at least 100mg). When the concentration of succinate was increased to 3.6mM, only 12–25% greater yields of 5-aminolaevulinate were obtained. Since this entailed a considerable loss of sensitivity and much greater amounts of labelled succinate were therefore required, the concentration of succinate was kept at 0.37mM throughout the present work. In some experiments conversion of [1,4-^{14}C]succinate was also studied.

5-Aminolaevulinate was obtained by ion-exchange chromatography and further purified by treatment with acetylacetone to form the pyrrole derivative 3-acetyl-4-(2-carboxyethyl)-2-methylpyrrole (Mauzerall & Granick, 1956) and by extracting the pyrrole into ethyl acetate at pH4.6 (Condie & Tephly, 1978). Overall recovery of 5-aminolaevulinate, determined by taking through the whole procedure a known amount of 5-amino[^{14}C]-laevulinate was in excess of 90%. The radioactive compound recovered in the ethyl acetate phase was compared with the pyrrole obtained from authentic 5-amino[^{14}C]laevulinate by determining (1) the ratio between the radioactivities recovered in the first and second ethyl acetate extracts of the eluate, (2) the ratio between the radioactivities present in the ethyl acetate before and after shaking it with an excess of unlabelled eluate of pH4.6 and (3) the recovery of radioactivity from silica gel 60 plates (0.25mm thick; E. Merck, Darmstadt, Germany), after t.l.c. in butanol/acetic acid (Mauzerall & Granick, 1956) and visual detection of the pyrrole spot by spraying with a mixture of modified Ehrlich/mercury reagent (Urata & Granick, 1963) and methanol (1:4, v/v). By all these criteria the radioactivity recovered in the ethyl acetate behaved as that obtained from authentic 5-aminolaevulinate and no significant contamination of the ethyl acetate extract by other radioactive compounds could be detected.

Portions (1ml) of the ethyl acetate extract were taken for counting radioactivity in a TriCarb model B 2450 liquid-scintillation spectrometer with Instagel as the scintillant, and correction for quench was by an external-standard/channel-ratio curve. Yields of 5-aminolaevulinate were calculated from the specific radioactivity of the [^{14}C]succinate at a 1:1 molar ratio. The radioactivities of blood plasma and of tissue homogenates from animals injected with [^{14}C]haematin were similarly determined and the contribution of blood plasma to the brain and liver radioactivity was calculated by assuming a 45% haematocrit value and a blood plasma content of 1.7% (Chiueh *et al.*, 1978) and of 7.7% (Cunningham *et al.*, 1976), for brain and liver respectively.

Source of special chemicals

Cycloheximide, bovine serum albumin (essentially fatty acid-free) and 5-aminolaevulinic acid (both the unesterified acid and its methyl ester) were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. 1,2-Propylene glycol (purum grade) was from Kebo AB, Stockholm, Sweden, and haemin samples

were generous gifts from Dr. S. Sassa, Rockefeller University, New York, NY, U.S.A., and from Dr. C. J. Watson, Northwestern Hospital, Minneapolis, MN, U.S.A. [1,4-^{14}C]Succinic acid (sp. radioactivity 51.3 Ci/mol), [2,3-^{14}C]succinic acid (sp. radioactivity 68.1 Ci/mol) and 5-amino[5-^{14}C]-laevulinic acid (sp. radioactivity 49 Ci/mol) were obtained from New England Nuclear G.m.b.H., Dreiechenhain, Germany.

Results

Conversion of [^{14}C]succinate into 5-aminolaevulinate and into unknown metabolite in vitro

In the first experiment, in which adult male rats were used, evidence was obtained that both liver and brain homogenates could convert [^{14}C]succinate into 5-aminolaevulinate *in vitro*, liver being considerably more active than brain (Table 1). This confirms previous findings (Paterniti *et al.*, 1978; Percy & Shanley, 1979; Maines, 1980). When the trichloroacetic acid-soluble supernatants from incubation of either tissue with [2,3-^{14}C]succinate were subjected to ion-exchange chromatography, the 1.0M-sodium acetate eluates contained, in addition to 5-aminolaevulinate, some other unknown metabolite(s). The amount of radioactivity present as unknown metabolite was relatively small with liver but very marked with brain, where it exceeded the radioactivity present as 5-aminolaevulinate by 50-fold. In spite of this large excess, complete separation of 5-aminolaevulinate from the unknown contaminant was achieved by a further purification step, as described in the Materials and methods section.

When [2,3-^{14}C]succinate and [1,4-^{14}C]succinate were compared as precursors of 5-aminolaevulinate, a 15–40% greater incorporation of the methylene carbon atoms of succinate was found with both liver and brain (Table 1), compared with the incorporation of the carboxy carbon atoms of succinate.

Table 1. *Conversion of [^{14}C]succinate into 5-aminolaevulinate by brain and liver homogenates in vitro: comparison between [2,3-^{14}C]- and [1,4-^{14}C]-succinate*

Brain and liver homogenates were incubated as described in the Materials and methods section with either [2,3-^{14}C]- or [1,4-^{14}C]-succinate (0.37mM). Results are those of individual observations, each obtained with the liver and brain from a different adult rat and are given as pmol of [^{14}C]-succinate converted into 5-aminolaevulinate/min per g wet wt. of tissue.

Labelled succinate	Succinate converted (pmol)	
	Brain	Liver
[2,3-^{14}C]	25, 25, 32	132, 145, 111, 134
[1,4-^{14}C]	15, 21, 21	116, 131, 88, 108

Postnatal development of 5-aminolaevulinate synthase in brain and liver

Previous work has shown an age-dependent maturation of several metabolic pathways in the brain, including the respiratory activity of brain (Land *et al.*, 1977) and its concentration of mitochondrial cytochromes (Chepelinsky & Arnaiz, 1970; Bull *et al.*, 1979). All of these components or activities are very low at birth, then steadily increase reaching the adult activity at approx. 20 days of age. In contrast, the activity of 5-aminolaevulinate synthase, which was also very low at birth, showed a

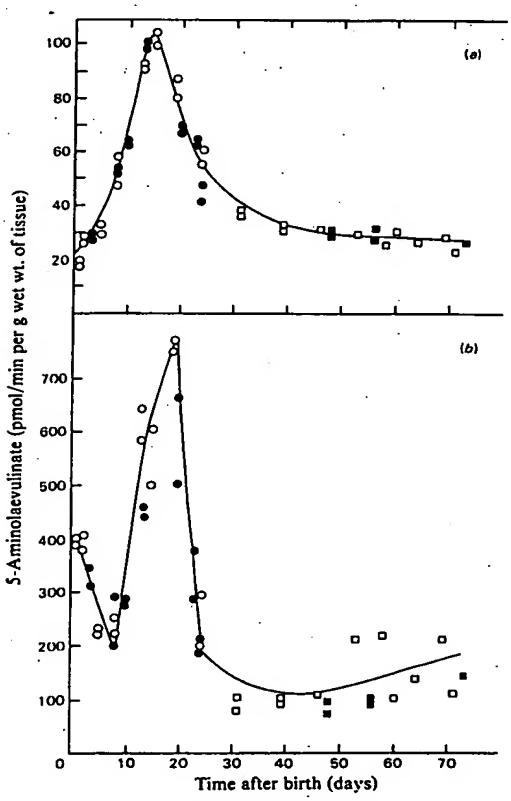

Fig. 1. *Postnatal development of brain and liver 5-aminolaevulinate synthase in the rat*

Male rats were killed at different times after birth and the conversion of [2,3-^{14}C]succinate into 5-aminolaevulinate by their brain (*a*) and liver (*b*) homogenates was determined. Each point represents the result obtained with the pooled homogenates from two rats. Different symbols represent batches of rats with a slightly different birth date. Note that the scales of the ordinates differ in (*a*) and (*b*).

rapid increase thereafter, reaching a peak at about 15 days and subsequently declining to the much lower adult value (Fig. 1*a*). Therefore there was a sharp peak of 5-aminolaevulinate synthase activity preceding and accompanying the increase in mitochondrial cytochromes, suggesting a physiological response to the increased haem demand.

The age-dependent behaviour of 5-aminolaevulinate synthase was somewhat different in rat liver, where activities at birth were relatively high and a peak of increased activity was noted (at about 20 days of age), after an initial decline (Fig. 1*b*). Both the initial decline in hepatic 5-aminolaevulinate synthase activity in the first few days after birth and the peak of increased activity at around 20 days of age have already been reported (Woods & Dixon, 1970; Maines & Kappas, 1978). In the liver, turnover of microsomal cytochromes, especially cytochrome *P*-450, accounts for most of the haem that is synthesized (Meyer & Schmid, 1977); this is in contrast with the situation in the brain, where most of the newly formed haem appears to be destined for the synthesis of mitochondrial cytochromes (Percy & Shanley, 1979). It is therefore noteworthy that in the liver the wave of increased 5-aminolaevulinate synthase (Fig. 1*b*) again precedes and accompanies the maturation (Song *et al.*, 1971) of the predominant haemoproteins, in this case the cytochromes of the *P*-450 group.

Effect of various treatments on brain and liver activities of 5-aminolaevulinate synthase in adult rats

The finding of increased brain 5-aminolaevulinate synthase at a time during development when haem requirements might be expected to be greater suggested that in brain, as in other organs (for example, the liver), this enzyme might regulate haem synthesis and be subjected to homoeostatic control. Several treatments were compared for their effect on the brain and liver enzymes. It was found (Table 2) that cycloheximide decreased the enzyme activity in both tissues, whereas intravenously administered haem (given in either of two different forms) or subcutaneously injected $CoCl_2$ decreased the enzyme activity only in the liver. Cycloheximide, $CoCl_2$ and haematin have already been reported to prevent the stimulation of hepatic 5-aminolaevu-

Table 2. *Effect of administering to rats either cycloheximide, $CoCl_2$, haematin or 5-aminolaevulinic acid on the conversion in vitro of [2,3-^{14}C]succinate into 5-aminolaevulinate by their brain and liver homogenates*

Male rats (240–320 g) were given various treatments by subcutaneous (s.c.) or intravenous (i.v.) injections as indicated and were killed 1 h or 2 h later. Control rats were injected with saline, albumin, Na_2CO_3 or propylene glycol solutions as appropriate. Conversion of [2,3-^{14}C]succinate into 5-aminolaevulinate was measured *in vitro* as described in the Materials and methods section. Results given are means ± S.E.M. of at least four observations. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$, when compared with corresponding controls of the same experiment, but injected with solvent alone. Further abbreviation used: N.D., not detectable in any of the four samples.

Experiment	Treatment and dose	Time of killing (h after treatment)	[2,3-^{14}C]Succinate converted into 5-aminolaevulinate (pmol/min per g wet wt. of tissue)	
			Brain	Liver
A	Saline (10 ml/kg body wt., s.c.)	1	31.1 ± 1.8	137.4 ± 15
	$CoCl_2,6H_2O$ (60 mg/kg body wt., s.c.)	1	32 ± 1.4	28.1 ± 9***
	Cycloheximide (40 mg/kg body wt., i.v.)	1	21.1 ± 2**	61.2 ± 10*
B	Albumin (1.25%, in saline) (0.8 ml/kg body wt., i.v.)	2	35 ± 0.41	129 ± 39
	Methaemalbumin (equivalent to 3.2 mg of haemin/kg body wt., i.v.)	2	38.5 ± 4.6	12.5 ± 2.4*
C	Na_2CO_3 (0.25%) (1.8 ml/kg body wt., i.v.)	2	33.9 ± 2.4	109 ± 24.4
	Haematin in Na_2CO_3 solution (equivalent to 7.5 mg of haemin/kg body wt., i.v.)	2	31.9 ± 1.9	11.3 ± 1.6**
D	Propylene glycol/saline (1 : 2, v/v) (0.8 ml/kg body wt., i.v.)	1	35.5 ± 1.5	72.7 ± 12
		2	33 ± 2.5	75 ± 16
	5-Aminolaevulinic acid (methyl ester) in propylene glycol/saline (22.8 mg/kg body wt., i.v.)	1	18.1 ± 1.4***	6.1 ± 1**
		2	14.7 ± 1.9**	3.1 ± 1**
	5-Aminolaevulinic acid (free acid) in propylene glycol/saline (21.2 mg/kg body wt., i.v.)	2	32.5 ± 1.3	N.D.

Table 3. *Radioactivity recovered at different times after injection of [^{14}C]haematin in blood plasma and homogenates of brain, liver, kidney and spleen of the rat*

Male rats were injected intravenously with [^{14}C]haematin and killed at different times thereafter. In experiment (*a*) rats (body wt. 129–139 g) were injected with methaemalbumin (equivalent to 4.9–5.3 mg of haemin/kg body wt.) and in experiment (*b*) rats weighed 278–284 g and were given hematin dissolved in 0.25% Na_2CO_3 at a dose of 6.7–7.4 mg of haemin/kg body wt. Results given refer to individual rats.

		Radioactivity recovered at 30 min after injection (d.p.m./g or d.p.m./ml)		Time course of radioactivity (% of that observed at 3 min)			
Experiment	Tissue	Observed	Corrected for entrapped plasma	3 min	15 min	30 min	90 min
(*a*)	Plasma	5885	—	100	58.8	29.4	16.5
	Brain	116	16	100	58.0	35.3	18.5
	Liver	3707	3254	100	190.2	187.0	185.8
	Kidney	1041	—	100	82.8	85.5	68.2
	Spleen	725	—	100	126.7	97.2	82.5
(*b*)	Plasma	16737	—	100	—	66.0	39.3
	Brain	309	25	100	—	67.7	44.4
	Liver	7175	5886	100	—	351.0	420.4
	Kidney	1348	—	100	—	99.3	114.0
	Spleen	1533	—	100	—	114.9	155.0

linate synthase caused by inducers (Hayashi *et al.*, 1969; Gayathri *et al.*, 1973; Maines *et al.*, 1976; De Matteis & Gibbs, 1977) and $CoCl_2$ also to cause a decrease in the basal activity of the liver enzyme (Nakamura *et al.*, 1975; Sinclair *et al.*, 1979).

An intravenous load with 5-aminolaevulinate did not affect markedly the brain synthase (even though it completely abolished the enzyme activity in the liver), but injection of similar amounts of the methyl ester of 5-aminolaevulinic acid caused a very significant decrease in the activity of the brain enzyme (Table 2). Previous studies have shown that parenterally injected 5-aminolaevulinic acid is taken up with difficulty by the brain but very effectively by the liver (McGillion *et al.*, 1975; Shanley *et al.*, 1976), and we suggest that the marked inhibition of the brain enzyme observed after a load of the methyl ester reflects passive diffusion of the unchanged methyl ester across the blood/brain barrier, followed by hydrolysis to the free amino acid within the brain and subsequent conversion of 5-aminolaevulinate into haem. Uptake of several amino acid esters (but not of the corresponding free amino acids) has also been reported with rat liver lysosomal preparations incubated *in vitro* and the methyl esters have been shown to undergo hydrolysis to the free amino acids within the lysosomes (Reeves, 1979).

The differential effect of Co^{2+} on 5-aminolaevulinate synthase in the two organs is also probably due to differential uptake, as the concentration of cobalt was found to be considerably higher in liver (13.5 μg/g wet wt. of tissue, on average) than in brain (<0.3 μg/g wet wt.) of treated animals. Evidence is provided below that the lack of effect of intravenously administered haematin on brain 5-aminolaevulinate synthase was similarly due to lack of uptake by brain tissue.

Fate of intravenously administered labelled haematin

Rats were injected with [^{14}C]haematin, either as methaemalbumin or as a solution in 0.25% (w/v) Na_2CO_3. The latter was also used as several investigators have claimed that haematin administered in this form is beneficial to patients suffering from the neurological symptoms of acute porphyria. No evidence for brain uptake of haematin could be obtained. For example, when methaemalbumin was injected (Table 3*a*), in agreement with previous findings (Snyder & Schmid, 1965), the liver radioactivity was in excess of that expected from contamination by entrapped plasma, but the radioactivity recovered in brain was very small and most of it could be accounted for by contaminating blood plasma. In addition, the time course of radioactivity suggested substantial uptake of label by liver (and possibly to some extent by kidney and spleen), whereas the time course of the brain radioactivity followed very closely that of plasma, again suggesting that the small amount of label present in brain is probably due to its blood plasma content. Similar findings were obtained with haematin dissolved in Na_2CO_3 solution (Table 3*b*).

Discussion

We have confirmed the presence in the brain of the first enzyme of the pathway of haem biosynthesis, 5-aminolaevulinate synthase, and also found that the enzyme undergoes changes in activity

at different stages of development from birth to adult age. The wave of increased activity seen in the first few weeks of life preceded and accompanied the formation of brain haemoproteins, suggesting a physiological response of the brain enzyme to ensure adequate supply of haem within the brain cells during a period of increased demand. This suggests that the brain can itself produce the haem required for the synthesis of its own haemoproteins and that it accomplishes this, like other organs, by increasing the activity of the rate-controlling enzyme of haem biosynthesis, 5-aminolaevulinate synthase.

In the adult rat both the concentration of the main haemoproteins of the brain (the mitochondrial cytochromes; see Bull *et al.*, 1979) and the activity of brain 5-aminolaevulinate synthase (Fig. 1*a*) are approximately in a steady state. The amount of haem, and therefore of 5-aminolaevulinate, required to maintain the turnover of brain cytochromes was calculated, using the total cytochrome content given by Bull *et al.* (1979) (46.8 nmol/g wet wt. of cortical slices) and assuming the same $t_{\frac{1}{2}}$ value (132 h), which has been reported (Aschenbrenner *et al.*, 1970) for the turnover of mitochondrial cytochromes in both liver and heart of the adult rat. The amount of 5-aminolaevulinate required (33 pmol/min per g of brain) is in good agreement with activity of 5-aminolaevulinate synthase observed in this work between 46 and 70 days of age [29 ± 0.8 pmol/min per g (mean ± S.E.M.); Fig. 1*a*]. This also suggests that the brain is capable of producing haem for its own needs and is not likely to depend on the liver (Maines, 1980; Bissell *et al.*, 1979) for its haem requirements.

This work has also shown that the brain synthase activity can be decreased, like the liver enzyme, by giving cycloheximide or by large doses of 5-aminolaevulinate, especially when this precursor of haem is administered as the methyl ester, presumably because it can then diffuse more readily across the blood/brain barrier and generate intercerebrally enough haem to exercise a feedback type of action on the enzyme. Paterniti *et al.* (1978) and Percy & Shanley (1979) have reported that brain 5-aminolaevulinate synthase is not affected by administration of either ethanol, 2-allyl-2-isopropylacetamide or 3,5-diethoxycarbonyl-1,4-dihydrocollidine, treatments all known to induce the liver enzyme, suggesting some basic difference in the way in which the enzyme is regulated in the two organs. Percy & Shanley (1979) have further suggested that an inability of brain 5-aminolaevulinate synthase to respond with induction may explain the neurological symptoms of acute porphyria, as the inherited enzymic block in the pathway of haem biosynthesis may not be compensated in the brain (as it is in the liver) by increased production of the haem precursors. Our observations would on the other hand suggest that brain 5-aminolaevulinate synthase may be regulated by haem as in other tissues and that the failure of inducers of the liver enzyme to stimulate the synthase in brain is due to them all acting indirectly, for example by interaction with the drug-metabolizing system (De Matteis, 1978), the activity of which is known to be very low in brain (Sasame *et al.*, 1977). More direct evidence that the brain 5-aminolaevulinate synthase is in fact inducible in the adult rat has recently been found (F. De Matteis & D. E. Ray, unpublished work).

Previous work (Shemin, 1955) has shown that succinate can be converted into 5-aminolaevulinate by two different pathways. In the first pathway succinyl-CoA, the immediate precursor of 5-aminolaevulinate, is generated directly by the enzyme succinyl-CoA synthetase (EC 6.2.1.5); in the second, succinate enters the tricarboxylic acid cycle and succinyl-CoA is generated from the oxidative decarboxylation of α-oxoglutarate. Since in the latter pathway both carboxy carbon atoms of succinate are lost, whereas both methylene carbon atoms are retained, incorporation of [1,4-^{14}C]succinate into 5-aminolaevulinate can only take place by the succinyl-CoA synthetase pathway. In contrast, incorporation of radioactivity from [2,3-^{14}C]-succinate will proceed by both pathways (Shemin, 1955). It can therefore be concluded from the results of Table 1 that both pathways operate in the brain [as they do in the erythropoietic system (Shemin, 1955) and in the liver (see also Granick & Urata, 1963)] and that under the standard conditions of our enzymic assay most of the succinate is utilized for 5-aminolaevulinate synthesis, via the succinyl-CoA synthetase pathway.

When radioactive [^{14}C]haematin was administered intravenously to rats, uptake of radioactivity by liver (and possibly by kidney and spleen) could be demonstrated, but no significant uptake was found in the case of brain. This indicates that the blood/brain barrier is impermeable to haematin and casts doubts on the hypothesis that exogenous haem might remedy a situation of brain haem deficiency during the acute attack of human porphyria. If haematin injections are in fact beneficial to these patients, they are more likely to act by suppressing excess formation of 5-aminolaevulinate in the liver and its subsequent conversion into the postulated neurotoxic principle.

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Regulation of 5-Aminolevulinate Synthase mRNA in Different Rat Tissues*

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Gopesh Srivastava, Iain A. Borthwick, Deborah J. Maguire‡, Cornelis J. Elferink, Michael J. Bawden, Julian F. B. Mercer§, and Brian K. May¶

From the Adelaide University Centre for Gene Technology, Department of Biochemistry, University of Adelaide, G. P. O. Box 498, Adelaide, South Australia, 5001, Australia, the ‡Institut für Molekularbiologie I, Universität Zürich, 8093 Zürich, Hönggerberg, Switzerland, and the §Murdoch Research Institute, Royal Children's Hospital, Flemington Road, Parkville, Victoria, 3052, Australia

cDNA clones for rat liver 5-aminolevulinate synthase have been isolated and used to examine mRNA levels in different rat tissues. Northern hybridization analysis of total RNA from various rat tissues showed the presence of a single 5-aminolevulinate synthase mRNA species of estimated length 2.3 kilobases. Primer extension and RNase mapping studies indicated that the mRNA is identical in all tissues. Highest basal levels were seen in liver and heart. Administration of hemin to rats reduced the basal level of this mRNA only in liver but the heme precursor, 5-aminolevulinate (or its methyl ester), repressed the basal levels in liver, kidney, heart, testis, and brain. The drug 2-allyl-2-isopropylacetamide increased the mRNA level in liver and kidney only while human chorionic gonadotropin hormone elevated the level in testis. Administration of the heme precursor 5-aminolevulinate prevented these inductions. Nuclear transcriptional run-off experiments in liver cell nuclei showed that 2-allyl-2-isopropylacetamide and 5-aminolevulinate exert their effect by altering the rate of transcription of the 5-aminolevulinate synthase gene. The results indicate that a single 5-aminolevulinate synthase mRNA is expressed in all tissues and that its transcription is negatively regulated by heme.

5-Aminolevulinate synthase catalyzes the first step of the heme biosynthetic pathway and in the liver at least is rate-limiting (reviewed in Refs. 1 and 2). The enzyme performs a housekeeping function since all animal cells synthesize their own heme for mitochondrial cytochromes and other cellular hemoproteins. The enzyme level is normally very low in animal tissues but is greatly elevated in the liver of experimental animals following administration of a wide variety of porphyrinogenic drugs such as AIA[1] and phenobarbital (3). This biochemical response mimics the acute porphyria diseases in man where hepatic 5-aminolevulinate synthase levels are elevated during clinical attacks. Drugs which precipitate such attacks induce 5-aminolevulinate synthase levels in experimental animals. These same drugs also induce the synthesis of hepatic cytochrome P-450 proteins, which are involved in the conversion of foreign compounds to water-soluble derivatives.

Granick (3) first demonstrated that the end product heme prevented the drug-induced increase in hepatic 5-aminolevulinate synthase enzyme levels. Work in our laboratory and elsewhere (reviewed in Ref. 1) has suggested that heme acts by repressing the synthesis of 5-aminolevulinate synthase mRNA, but there has been no direct proof of this. Current evidence favors the hypothesis that the porphyrinogenic drugs act by inducing synthesis of cytochrome P-450 apoprotein which results in a reduction in the heme concentration, thus indirectly leading to an increase in 5-aminolevulinate synthase mRNA levels (1). Erythroid 5-aminolevulinate synthase is not induced by porphyrinogenic drugs (2), and this finding has led to the proposal that erythroid and hepatic 5-aminolevulinate synthases are distinct enzymes (4, 5). Indeed, it has been proposed that a multigene family exists for 5-aminolevulinate synthase with different mRNA species synthesized in different tissues (6). However, recent work suggests that 5-aminolevulinate synthase mRNA is the same in the liver and erythroid spleen of mice (7).

We are interested in determining at the molecular level how heme regulates the gene for 5-aminolevulinate synthase in liver and other tissues. cDNA (8, 9) and genomic clones (10) for chicken 5-aminolevulinate synthase have been isolated in this laboratory. In this communication we report the isolation of cDNA clones for rat liver 5-aminolevulinate synthase and provide strong evidence that the mRNA is identical in all rat tissues examined. We have established that drugs increase the level of 5-aminolevulinate synthase mRNA in a tissue-specific fashion and that heme represses levels of this mRNA in all tissues examined except erythroid spleen. Unequivocal evidence has been obtained that the altered levels of hepatic 5-aminolevulinate synthase mRNA observed after drug or heme administration are due to changes in the rate of transcription of the gene.

EXPERIMENTAL PROCEDURES

Materials—AIA was a generous gift from Roche, Australia. Hemin (ferriprotoporphyrin IX chloride) was supplied by Porphyrin Products, Logan, UT. A chicken β-actin cDNA clone in pBR322 (insert 1.8 kb) was provided by S. Dalton and a chicken serum albumin cDNA clone in pBR322 (insert 2 kb) by A. H. Hobbs. All other materials were purchased from sources previously described (9, 11).

Treatment of Animals—Male albino Wistar rats (200 g body weight) were given injections of AIA (80 mg) subcutaneously, 5-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J03190.

¶ To whom correspondence should be addressed.

[1] The abbreviations used are: AIA, 2-allyl-2-isopropylacetamide; SDS, sodium dodecyl sulfate; HCG, human chorionic gonadotropin hormone; RNase, ribonuclease; kb, kilobase(s); bp, base pair(s).

aminolevulinate or its methyl ester (333 mg) via the intraperitoneal cavity, or hemin (1.5 mg) via the tail vein. For induction with AIA over 12 h, a second injection of AIA was given at 7 h. 5-Aminolevulinate or hemin was given immediately after AIA injection. Rats were treated with HCG over 48 h by repeated subcutaneous injections (20 units) at 12, 24, and 36 h; 5-aminolevulinate was administered at 36 h and total mRNA isolated after a further 12 h. To obtain anemic rats, phenylhydrazine HCl (1.5 mg) was injected subcutaneously on 5 consecutive days and mRNA isolated on the sixth day. Anemic rats were treated with 5-aminolevulinate 12 h prior to death.

For developmental studies, Sprague-Dawley rats were used. Fetal ages were estimated from the time of mating; males were placed with females overnight and the following morning taken as day 0 of gestation. Total RNA was isolated essentially by the method of Liu *et al.* (12) from pooled individuals of at least 2 litters for each age.

Construction and Screening of a Rat Liver cDNA Library—Liver poly(A)$^+$ RNA from rats treated with AIA was used to construct a cDNA library. Double-stranded cDNA was synthesized by the procedure of Gubler and Hoffman (13), and DNA greater than 1600 bp was annealed with dG-tailed *Pst*I-digested pBR322 and transformed into *Escherichia coli* MC1061. Recombinant plasmids were screened with a mixture of ^{32}P nick-translated probes comprising the four *Pst*I inserts from the chicken 5-aminolevulinate synthase clone, p105B1 (9).

The clone p101B1 was sequenced by digestion of plasmid with either *Hpa*II, *Pst*I, *Sau*3A1, or *Taq*I and fragments sequenced by the method of Sanger *et al.* (14).

Analysis of RNA—Total RNA was isolated from rat tissues by the guanidine hydrochloride extraction procedure of Brooker *et al.* (15). Poly(A)$^+$ mRNA was prepared by oligo(dT)-cellulose chromatography.

For Northern blot analysis, RNA was electrophoresed in 1.0% agarose gels containing 1.1 M formaldehyde as described (11). RNA was transferred to nitrocellulose filters (BA85 from Schleicher & Schuell) and hybridized to the ^{32}P nick-translated rat 5-aminolevulinate synthase cDNA clone p101B1 (10 ng/ml) in a solution containing 50% formamide, 5 × SSPE (0.9 M NaCl, 50 mM sodium phosphate buffer, pH 7.0, 5 mM EDTA), 5 × Denhardt's (0.04% Ficoll, 0.04% polyvinylpyrrolidone, 0.04% bovine serum albumin), 0.1% SDS, 0.5% sodium pyrophosphate, and 200 μg/ml denatured salmon sperm DNA at 42 °C for 20 h. Filters were washed finally in 0.1 × SSPE containing 0.1% SDS at 60 °C for 40 min. For low stringency conditions filters were hybridized as above and washed in a solution containing 2 × SSPE and 0.1% SDS at 50 °C for 40 min. Molecular size markers consisted of DNA fragments generated by *Acc*I digestion of pBR322.

RNA was denatured and bound to nitrocellulose filters using either a slot or dot blot apparatus (Schleicher & Schuell). Hybridization conditions were as described above. The amounts of RNA in Northern and slot blots were quantitated using an LKB laser densitometer.

Primer extension analysis using poly(A)$^+$ RNA from different rat tissues and 5′-^{32}P-labeled synthetic primers complementary to the coding strand of p101B1 was carried out according to the method of McKnight *et al.* (16). The extended products were analyzed by electrophoresis on 8 M urea, 6% polyacrylamide gels with a dideoxy sequence ladder of M13 bacteriophage DNA as size standards or ^{32}P-labeled *Hpa*II fragments from pBR322.

RNase Mapping—The three *Pst*I restriction fragments of p101B1 (see Fig. 5) were individually subcloned into *Pst*I cut pGEM-1 vector (Promega Biotech). Two probes, A and E, contained the 5′ and 3′ *Pst*I fragments of p101B1, respectively (see Fig. 5). The plasmid containing the largest *Pst*I fragment of p101B1 was further digested with appropriate restriction enzymes to generate three subclones of suitable size for RNase mapping; restriction enzyme removal of a *Bam*HI fragment (one *Bam*HI site in polylinker) generated a clone for the protection of a 364-bp *Bam*HI-*Pst*I fragment (probe D). Digestion of the plasmid with *Bgl*II and *Hin*dIII (polylinker site) allowed the religation of a clone for the protection of a 631-bp *Pst*I-*Bgl*II fragment (probe B). Probe C was generated by directionally cloning the 699-bp *Bam*HI/*Sal*I fragment into a *Bam*HI/*Sal*I cut pGEM1 vector.

RNA probes uniformly labeled with [^{32}P]UTP were generated *in vitro* from the five recombinant pGEM plasmids using either T7 or SP6 polymerase as described (11). Specific activities of about 10^9 cpm/μg RNA were routinely obtained. Full-length transcripts were isolated on a 5% polyacrylamide sequencing gel and eluted in 500 mM ammonium acetate, 1 mM EDTA, 0.5% SDS for 3–6 h at 37 °C. RNase mapping using RNase A and T1 was carried out as described previously (11) and protected fragments analyzed following electrophoresis on a 5% polyacrylamide sequencing gel and autoradiography.

Nuclear Transcription Assays—Nuclei were isolated from rat liver as described by Schibler *et al.* (17). The transcription reactions contained 100 mM Tris-HCl, pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 1.5 mM $MnCl_2$, 0.4 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1.2 mM dithiothreitol, 30% glycerol, 2 μM UTP, 1 mM each of ATP, CTP, and GTP, 100 μCi of [α-^{32}P]UTP, and 1.5 × 10^7 nuclei in a final volume of 150 μl. These were incubated at 26 °C for 15 min, and ^{32}P-labeled RNA was extracted as described by Vannice *et al.* (18). Rats were induced with AIA for 4 h. When 5-aminolevulinate was administered, it was injected 10 h prior to AIA treatment and the nuclei prepared 4 h later. For quantitation of specific transcripts, 5 μg of the appropriate cloned DNAs (double-stranded DNA from the chicken serum albumin and β-actin cDNA clones or single-stranded DNA from an M13 phage clone containing the 1.7-kb *Pst*I fragment of p101B1) were denatured and applied to a nitrocellulose filter using a slot blot apparatus. Filters were prehybridized in 1 ml of 50% formamide, 5 × SSC, 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.1% sodium pyrophosphate, 0.1% SDS, 100 μg/ml *E. coli* tRNA, and 0.2% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin at 52 °C overnight. Hybridization was carried out in the same solution with 2 × 10^6 cpm of ^{32}P-labeled RNA for 72 h at 52 °C. Filters were washed twice at room temperature for 30 min in 2 × SSC, 0.1% SDS, 0.1% sodium pyrophosphate and then twice in 0.5 × SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 65 °C for 60 min. The hybridization signals were quantitated by laser densitometric scanning.

RESULTS

Isolation of Rat Liver 5-Aminolevulinate Synthase cDNA Clones—A cDNA library was constructed using poly(A)$^+$ RNA from livers of rats induced with the porphyrinogenic drug AIA. Size-selected double-stranded cDNA was used to construct a library of 4800 recombinant clones which were screened using a mixture of ^{32}P-labeled cDNA probes prepared from the four *Pst*I fragments of a previously isolated chicken liver 5-aminolevulinate synthase cDNA clone (9). Four clones gave positive hybridization signals, and the largest of these, p101B1, was sequenced. The sequence contained an open reading frame of 1929 nucleotides from nucleotides 17 to 1945 giving a predicted protein of 642 amino acids (Fig. 1). The sequence of the first 15 N-terminal amino acids of mature 5-aminolevulinate synthase purified from the mitochondria of drug-induced rat liver (19) was determined and shown to be identical to that deduced from the nucleotide sequence of p101B1 from position 185 to 229. This shows that the glutamine (at nucleotide 185) is the N-terminal amino acid of the mature protein. Upstream from this codon there are three in-frame d(ATG) codons. The d(ATG) codon at nucleotide 17 is assumed to be the initiation codon since it would result in the translation of a 5-aminolevulinate synthase precursor with a presequence of size 6 kDa in agreement with the size estimated from previous studies (19).

The deduced protein sequence of rat liver 5-aminolevulinate synthase precursor was compared with that of chicken (9) and mouse (7). The sequence of rat precursor was very similar to that of chicken, both in the N-terminal presequence (56 amino acids) and over most of the mature protein sequence. Surprisingly, the rat protein sequence showed less overall homology to the mouse enzyme, and indeed no homology existed within the presequence segments.

Northern Analysis of 5-Aminolevulinate Synthase mRNA—The size of 5-aminolevulinate synthase mRNA in different rat tissues was determined by Northern blot analysis. Total RNA was isolated from the liver, kidney, brain, and testis of untreated rats. Tissues were also examined following treatments which are known to elevate 5-aminolevulinate synthase activity levels; for this total RNA was isolated from AIA-treated rat liver and kidney, HCG-treated rat testis, and spleen of rats rendered anemic by phenylhydrazine treatment.

The RNA samples were fractionated on a formaldehyde

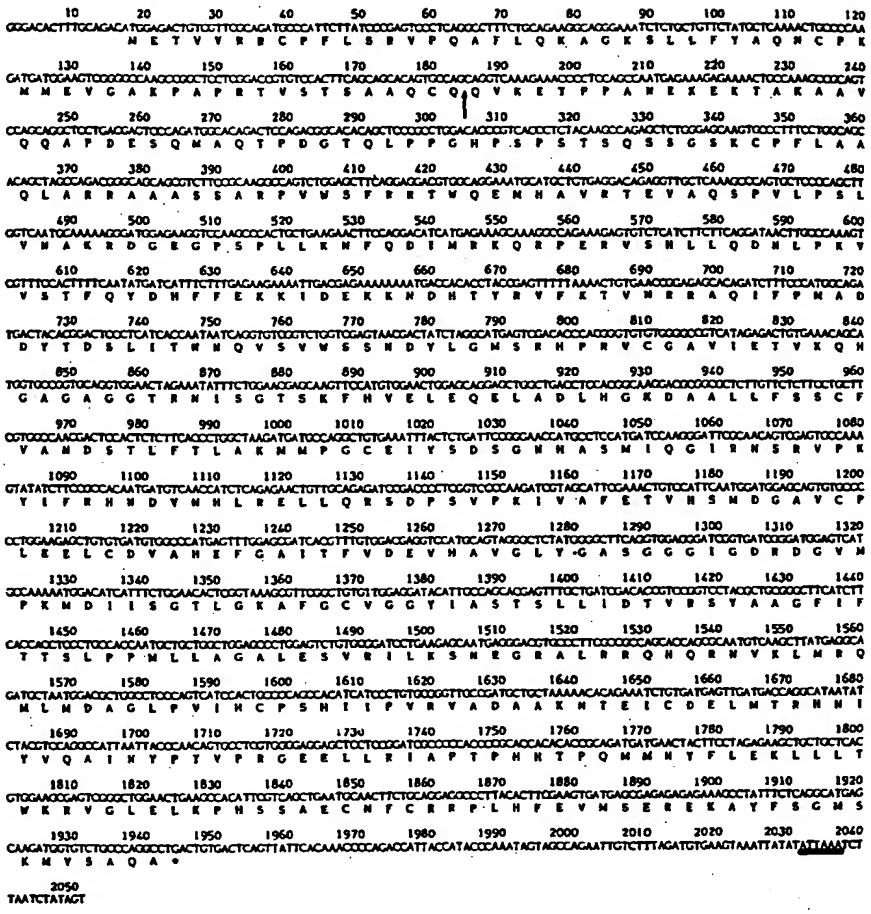


FIG. 1. **Nucleotide and predicted amino acid sequence of rat liver 5-aminolevulinate synthase.** The clone has 5′- and 3′-noncoding regions of 16 and 112 nucleotides, respectively. A possible polyadenylation signal ATTAAA is *underlined*, and the termination codon is *asterisked*. The *arrow* indicates the cleavage site of the presequence.

agarose-denaturing gel and probed with ^{32}P-labeled p101B1. In all tissues, only a single 5-aminolevulinate synthase mRNA species was detected, estimated to be 2.3 kb in length (Fig. 2). In some tracks, very faint extra bands were visible and were probably due to ribosomal RNA. The results therefore show that there is no difference in size between 5-aminolevulinate synthase mRNA from either untreated or induced rat tissues.

It was also of interest to determine the 5-aminolevulinate synthase mRNA during fetal development. A single 5-aminolevulinate synthase mRNA species (2.3 kb in length) was detected at day 14 of gestation when the liver is largely erythroid in nature (20) through to the adult stage (Fig. 3). The level of mRNA peaked prior to birth at gestation days 20 and 21. This level then declined significantly at birth and in the adult had returned to the level seen prior to birth.

Primer Extension and RNase Mapping Studies of 5-Aminolevulinate Synthase mRNA from Rat Tissues—Further studies were carried out to determine whether 5-aminolevulinate synthase mRNA was identical in different tissues of rat. Of particular interest was the relationship of the nonerythroid to erythroid mRNA in view of the proposal that these mRNAs are distinct in the chicken (6). A 5′-γ-^{32}P-labeled 23-nucleotide oligomer complementary to the coding sequence of p101B1 from nucleotides 17 to 40 (Fig. 1) was used in a primer extension reaction on poly(A)$^+$ RNA isolated from tissues of untreated and treated rats (see legend to Fig. 4). With all mRNA samples, two bands, 144 and 147 nucleotides in length, were seen following electrophoresis of the extension products and detection by autoradiography (Fig. 4). Such doublets have been observed previously and may be due to an artifact of the reverse transcriptase reaction (21) or to initiation of mRNA synthesis at an alternative nucleotide. A second set of primer extension reactions was performed using a primer complementary to a sequence in the 5′-untranslated region of p101B1 from nucleotides 3 to 26. The rationale for this was that different mRNAs in different tissues may be homologous in their coding sequences but different in the 5′-untranslated region. With all tissues a doublet was observed with bands 108 and 111 nucleotides in length (result not shown). The results suggest that the 5′-untranslated region of the 5-aminolevulinate synthase mRNA from different tissues is the same.

As indicated earlier, the 5′-untranslated region of p101B1 is 16 nucleotides in length. The primer extension analysis indicates that in the mRNA, this region is 100 or 103 nucleotides in length, and p101B1 is, therefore, 84 or 87 nucleotides short of being full-length.

For RNase mapping experiments, five [α-^{32}P]UTP-labeled RNA probes complementary to rat hepatic 5-aminolevulinate synthase mRNA were synthesized by *in vitro* transcription of pGEM-1 plasmids containing appropriate restriction fragments of p101B1 (see Fig. 5). The probe sequences (*A–E*)

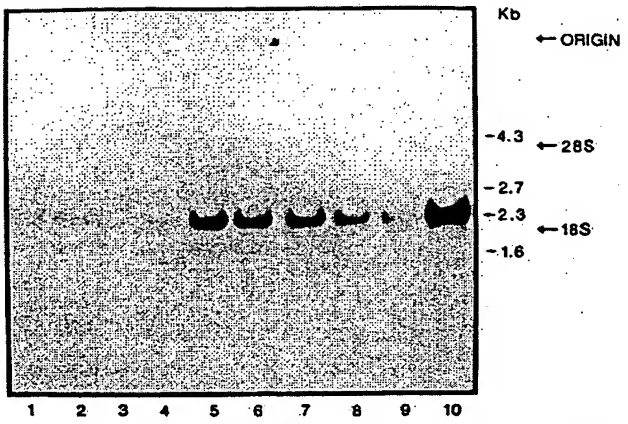


FIG. 2. Northern blot analysis of 5-aminolevulinate synthase mRNA in rat tissues. Total RNA from tissues of untreated or treated rats was electrophoresed on a formaldehyde/agarose gel and hybridized to nick-translated p101B1. Molecular size markers consisted of DNA fragments generated by *Acc*I digestion of pBR322. The positions of rRNA markers are shown. *Lane 1*, erythroid spleen (50 μg); *lane 2*, brain (50 μg); *lane 3*, testis (20 μg); *lane 4*, testis (HCG-treated rat) (10 μg); *lane 5*, heart (20 μg); *lane 6*, heart (AIA-treated rat) (20 μg); *lane 7*, kidney (AIA-treated rat) (20 μg); *lane 8*, kidney (50 μg); *lane 9*, liver (5 μg); *lane 10*, liver (AIA-treated rat) (5 μg).

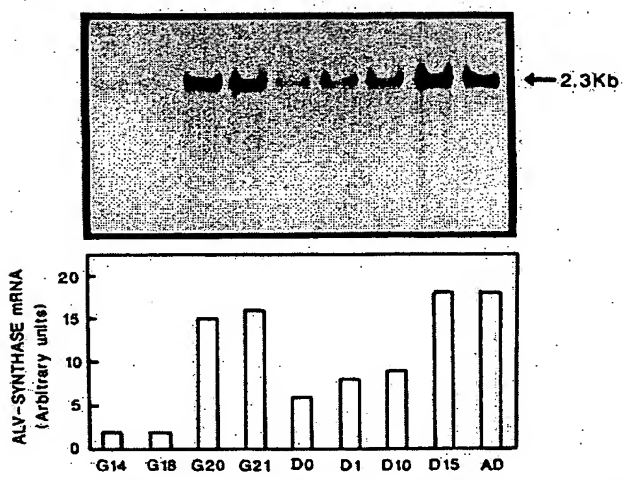


FIG. 3. Developmental profile of 5-aminolevulinate (*ALV*) synthase mRNA. Total RNA (10 μg) from rat liver at various stages of development was resolved on a formaldehyde/agarose gel and hybridized to nick-translated p101B1. The size of the mRNA was estimated as in Fig. 2, and mRNA levels were quantitated from the Northern blot by densitometric scanning and shown as *bar graphs*. Loadings of RNA were shown to be uniform by ethidium bromide staining of the gel. *G*, gestation day; *D*, day following birth; *AD*, adult, 12 weeks.

complementary to the mRNA were 73, 631, 699, 364, and 210 nucleotides in length, respectively, and spanned the entire mRNA except for 83 nucleotides not present in the cDNA clone at the extreme 5′-end and 90 nucleotides between the *Bgl*II and *Sal*I sites (see Fig. 5). Poly(A)$^+$ RNA samples were hybridized to these probes, and nonhybridized RNA was digested with RNase A and T1. The protected radiolabeled fragments were resolved on a 5% polyacrylamide sequencing gel.

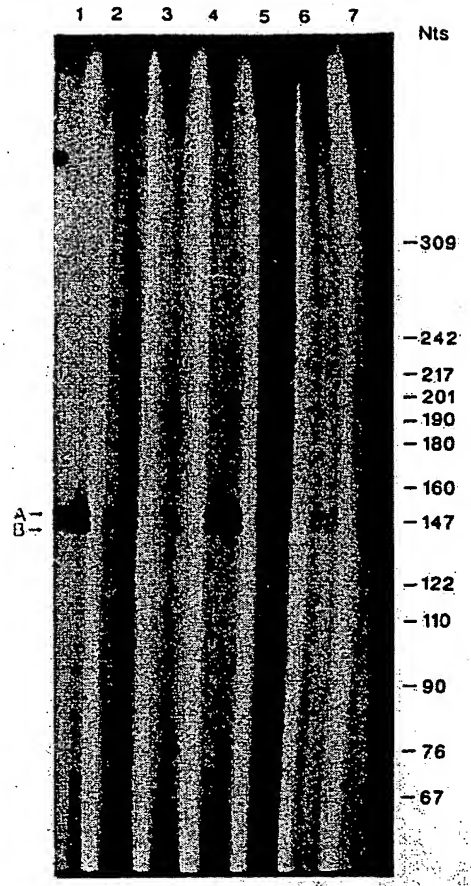


FIG. 4. Primer extension analysis of 5-aminolevulinate synthase mRNA in various rat tissues. A chemically synthesized 23-nucleotide oligomer was 5′-phosphorylated with [γ-^{32}P]ATP and used to primer extend on poly(A)$^+$ RNA from untreated rat liver (10 μg) (*lane 2*); brain (50 μg) (*lane 3*); heart (10 μg) (*lane 4*); erythroid spleen (50 μg) (*lane 7*); AIA-treated rat liver (5 μg) (*lane 1*); kidney (10 μg) (*lane 5*); HCG-treated rat testis (10 μg) (*lane 6*). Products were analyzed on an 8 M urea, 6% polyacrylamide gel with ^{32}P-labeled *Hpa*II fragments from pBR322 as size standards. *Bands A* and *B* are 147 and 144 nucleotides in length, respectively. *Nts*, nucleotides.

For liver and erythroid spleen mRNA, all five RNA probes were employed. Fragments representing full-length protection products for each probe were observed, establishing that these mRNAs are very likely identical. Mapping with probe B which spans the 3′-noncoding end of the liver mRNA revealed two bands smaller than the expected full-length product of 210 nucleotides (Fig. 5). These additional bands may be caused by heterogeneity in the lengths of the poly(A) tails of isolated liver mRNA. In other experiments only probe B was used with mRNA from liver, kidney, brain, heart and testis of untreated rats, from kidney of AIA-treated rats, and from testis of HCG-treated rats. This probe fully protected the expected 631-nucleotide fragment in all the mRNA samples examined (results not shown). In summary, this study, together with Northern blot and primer extension analyses, provides compelling evidence that 5-aminolevulinate synthase mRNA is identical in all rat tissues.

Measurement of Basal Levels of 5-Aminolevulinate Synthase mRNA in Rat Tissues—Total RNA was isolated from tissues of untreated rats, and 5-aminolevulinate synthase mRNA

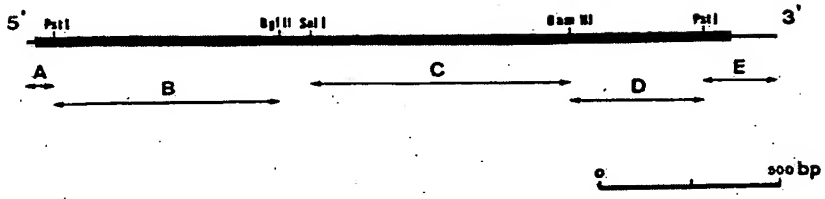
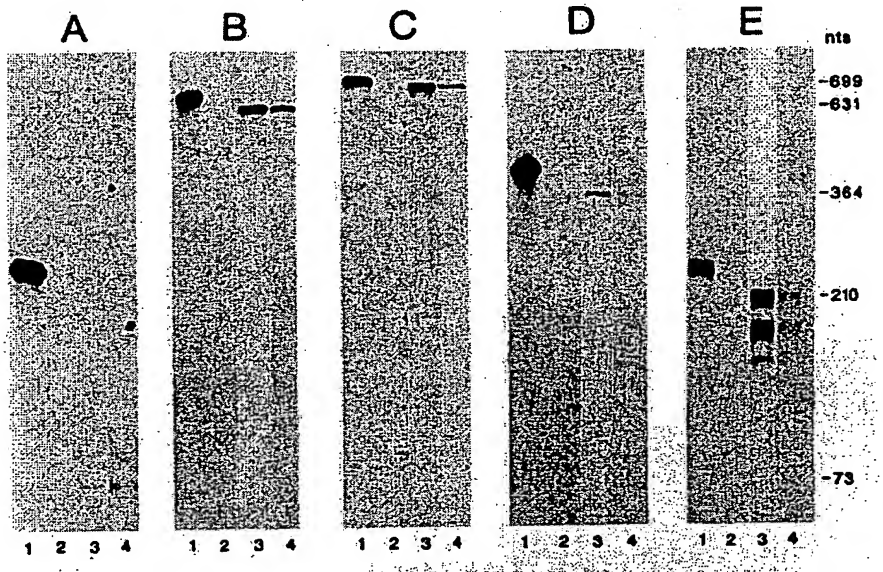
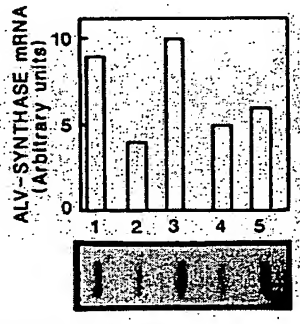


FIG. 5. RNase mapping of rat liver and erythroid spleen 5-aminolevulinate synthase mRNA. The cDNA clone, p101B1, is represented in the upper part of the figure with the coding and noncoding regions depicted as *heavy* and *light lines*, respectively. The *double-headed arrows* below show the relationship of the RNA probes *A–E* to p101B1. ^{32}P-Labeled RNA probes were hybridized to 2.5 μg of poly(A)$^+$ RNA from AIA-induced liver (*lane 3*) or erythroid spleen (*lane 4*) and incubated with RNase A and T1. Probes incubated in the absence of poly(A)$^+$ RNA were either untreated (*lane 1*) or treated (*lane 2*) with RNase A and T1. Protected fragments were analyzed by electrophoresis and autoradiography as described under "Experimental Procedures." The numbers displayed to the right of *panel E* correspond to the expected nucleotide lengths of the protected fragments. *nts*, nucleotides.





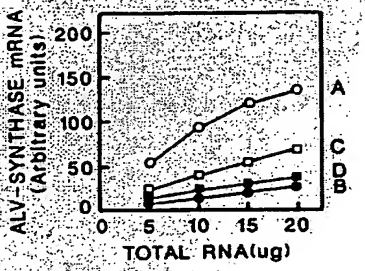


FIG. 6. Measurement of basal 5-aminolevulinate (*ALV*) synthase mRNA levels in rat tissues. Total RNA (10 μg) from tissues of untreated rats was applied as slots on nitrocellulose and hybridized to the nick-translated *PstI* inserts of p101B1. 5-Aminolevulinate synthase mRNA levels were quantitated by densitometric scanning and shown as *bar graphs*. *Lane 1*, liver; *lane 2*, kidney; *lane 3*, heart; *lane 4*, brain; *lane 5*, testis.

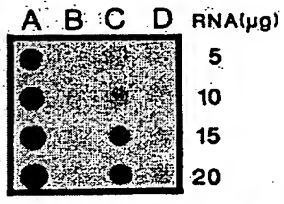


FIG. 7. Effect of hemin on 5-aminolevulinate (*ALV*) synthase mRNA levels in rat liver. Total RNA was isolated from rat liver 2 h after administration of AIA, hemin, or both. Amounts of total RNA (5–20 μg) were applied as dots on nitrocellulose and hybridized to the nick-translated *PstI* inserts of p101B1. mRNA levels were quantitated by densitometric scanning. *Lane A*, AIA treated; *lane B*, AIA and hemin treated; *lane C*, untreated; *lane D*, hemin treated.

amounts were quantitated. As can be seen in Fig. 6, the heart has the highest level of 5-aminolevulinate synthase mRNA, slightly above that of the liver, while in kidney, brain, and testis there is approximately half this level.

Effect of Hemin and 5-Aminolevulinate on 5-Aminolevulinate Synthase mRNA Levels in Untreated and Drug-treated Rats—The effect of hemin on liver 5-aminolevulinate synthase mRNA levels was first examined. Fig. 7 shows that

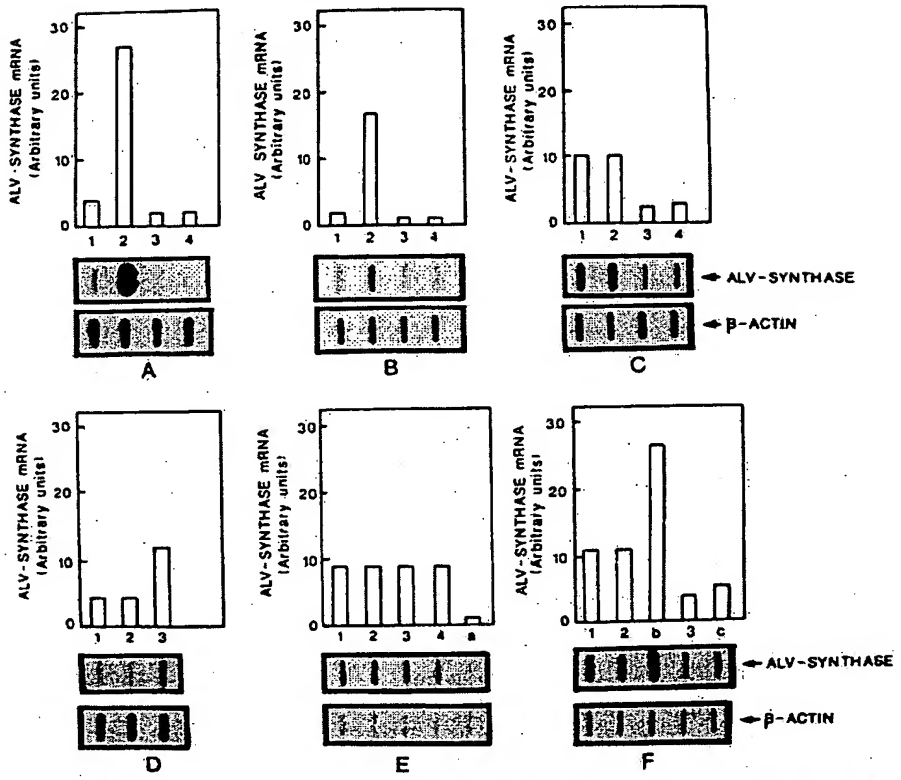


FIG. 8. **Measurement of 5-aminolevulinate (*ALV*) synthase mRNA levels in rat tissues after 5-aminolevulinate treatment.** Total RNA (10 μg) from tissues 12 h after administration of AIA or 5-aminolevulinate and from testis after HCG administration was applied as slots on nitrocellulose and hybridized to the nick-translated *Pst*I inserts of p101B1. mRNA levels were quantitated by densitometric scanning and shown as bar graphs. *Panel A*, liver; *panel B*, kidney; *panel C*, heart; *panel D*, erythroid spleen; *panel E*, brain; *panel F*, testis. *Lane 1*, untreated; *lane 2*, AIA treated; *lane 3*, 5-aminolevulinate treated; *lane 4*, AIA and 5-aminolevulinate treated; *lane a*, 5-aminolevulinate-methyl ester treated; *lane b*, HCG treated; *lane c*, HCG and 5-aminolevulinate treated.

treatment of rats with the drug AIA over a 2-h period increased the level of 5-aminolevulinate synthase mRNA (Fig. 7, *A* and *C*). Administration of hemin either to drug-treated or untreated rats reduced the level of 5-aminolevulinate synthase mRNA to below that of basal levels (Fig. 7, *B* and *D*).

In similar studies it was found that hemin had no effect on the mRNA level in the extrahepatic tissues investigated (data not shown). It was possible, however, that injected hemin was not reaching or not entering the cells of these tissues. Anderson *et al.* (22) has reported that administered 5-aminolevulinate is taken up by many tissues and converted rapidly to heme. The effect of 5-aminolevulinate on 5-aminolevulinate synthase mRNA levels in different rat tissues was, therefore, studied in both normal and drug-treated animals. Of the tissues studied only liver and kidney showed induction by AIA; 5-aminolevulinate administration completely prevented the increase in mRNA levels in both tissues (Fig. 8, *panels A* and *B*). Similarly, basal levels of 5-aminolevulinate synthase mRNA in the liver, kidney, heart, and testis were reduced by 5-aminolevulinate to low levels (Fig. 8). In the case of brain, 5-aminolevulinate had no effect, but the methyl ester derivative of 5-aminolevulinate significantly reduced the mRNA level (Fig. 8, *panel E*) presumably because this compound can readily cross the blood-brain barrier. Erythroid spleen was an exception with 5-aminolevulinate treatment resulting in an increase in 5-aminolevulinate synthase mRNA levels (Fig. 8, *panel D*).

In other experiments, it was shown that 5-aminolevulinate prevented the HCG induction of 5-aminolevulinate synthase mRNA in the testis (Fig. 8, *panel F*).

In all the experiments described here the level of β-actin mRNA in the tissues was quantitated (see Fig. 8, but not shown graphically) and was found to be essentially unchanged indicating that the response of 5-aminolevulinate synthase mRNA levels to AIA, HCG, or 5-aminolevulinate did not reflect a general cellular event. We also measured 5-aminolevulinate synthase activity in homogenates of all tissues examined above, and the amounts detected correlated closely with the changes observed in 5-aminolevulinate synthase mRNA levels (results not shown).

Hemin Acts at the Transcriptional Level in the Liver—The experiments described above show that in different tissues AIA and heme regulate the levels of 5-aminolevulinate synthase mRNA. We investigated whether, in liver, this reflected transcriptional control. Rat livers were removed, nuclei isolated, and gene transcription activities quantitated. In this *in vitro* system, it was established that incorporation by the nuclei of [α-^{32}P]UTP into total RNA was linear for at least 30 min, and the extent of incorporation was similar in nuclei from untreated rats and rats treated with AIA, 5-aminolevulinate, or both. Additionally, α-amanitin (2 μg/ml) in the reaction mixture inhibited total RNA synthesis by about 40% and completely inhibited the synthesis of specific transcripts.

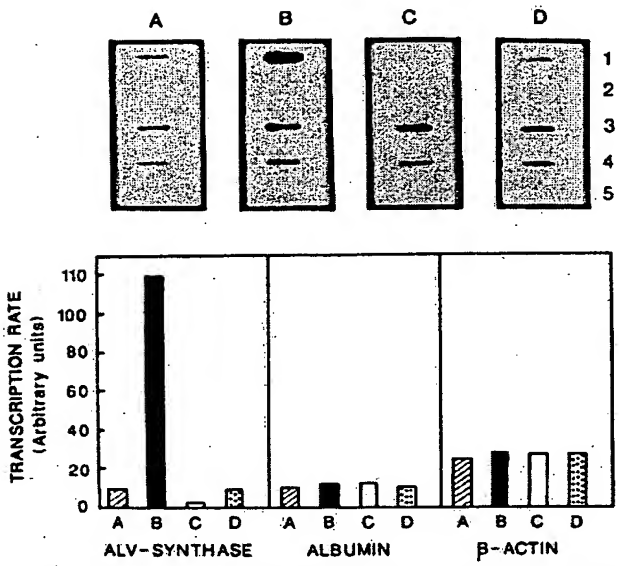


FIG. 9. **Effect of 5-aminolevulinate on 5-aminolevulinate (*ALV*) synthase gene transcription.** Rats were treated with AIA for 4 h. 5-Aminolevulinate was administered 10 h prior to AIA, and [^{32}P]RNA subsequently was isolated from rat liver nuclei and hybridized to nitrocellulose filter-bound cloned DNA. Transcription rates were quantitated from the slot blots by densitometric scanning and densities in arbitrary units shown as *bar graphs* after correction for the appropriate vector background controls. *A*, untreated rats; *B*, AIA treated; *C*, 5-aminolevulinate treated; *D*, AIA and 5-aminolevulinate treated. DNA clones: *lane 1*, 5-aminolevulinate synthase; *lane 2*, M13mp19; *lane 3*, chicken βactin; *lane 4*, chicken serum albumin; *lane 5*, pBR322.

Our results show that administration of AIA alone for 12 h resulted in a 10-fold increase in the transcriptional rate of the 5-aminolevulinate synthase gene (see Fig. 9). This correlated with a 7-fold increase in hepatic 5-aminolevulinate synthase mRNA levels measured at this time (see Fig. 8, *panel A*).

The effect of 5-aminolevulinate on the transcription rate of the 5-aminolevulinate synthase gene was investigated. Administration of this compound to untreated rats significantly reduced the low basal transcriptional rate of the 5-aminolevulinate synthase gene (Fig. 9). Administration of 5-aminolevulinate to AIA-treated rats prevented the drug-induced increase in the rate of gene transcription (Fig. 9). A corresponding reduction in the level of hepatic 5-aminolevulinate synthase mRNA was observed (Fig. 8, *panel A*). Throughout this work, the transcriptional rate of the serum albumin gene measured as a control was unchanged although that of the β-actin gene was slightly elevated by either AIA or 5-aminolevulinate treatment (Fig. 9). The reason for the latter is unknown. Other control experiments established that the presence of 5-aminolevulinate (50 μM) or hemin (0.01–10 μM) in the transcription reaction had no effect on any of the genes under test. Overall, these results demonstrate that in the liver, AIA and 5-aminolevulinate treatment modulates 5-aminolevulinate synthase mRNA levels by altering the rate of gene transcription.

DISCUSSION

The early work of Granick (3) established that in chick embryos drug inducibility of hepatic 5-aminolevulinate synthase activity is prevented by the simultaneous administration of heme. The mechanism of this has remained a central question ever since. It was originally postulated that heme and drugs compete for a site on a gene-controlling protein. This was rendered improbable by the work of Srivastava *et al.* (23) who showed that heme repression appeared to be the sole control and that drug induction was probably a secondary consequence of heme removal (reviewed in Ref. 1). Since the original postulate, it has been variously claimed that heme works at the translational and transcriptional level, and considerable confusion has existed (1). This was, in part, due to studies being done at the enzymic level and to the complication that hemin prevents entry of newly synthesized 5-aminolevulinate synthase into the mitochondrion (1).

In this work we have studied 5-aminolevulinate synthase control at the mRNA level. To this end a cDNA clone for rat liver 5-aminolevulinate synthase has been isolated and sequenced. Using probes derived from this, control of 5-aminolevulinate synthase mRNA in various rat tissues has been examined.

The first question investigated was whether there exists in rat a single mRNA for 5-aminolevulinate synthase or a multiplicity of them. This question arises from the suggestion that a family of 5-aminolevulinate synthase genes exists in chicken (6). The results here give compelling evidence that the 5-aminolevulinate synthase mRNA in all rat tissues examined is the same and that only a single species exists. This is in keeping with our recent conclusion that the erythroid 5-aminolevulinate synthase in chicken is coded for by the same gene as that in the liver (11). The work also shows that the rat liver 5-aminolevulinate synthase mRNA present during fetal development is indistinguishable on Northern blots from the adult form.

Although drug induction of 5-aminolevulinate synthase in liver is well known, the inducibility in other tissues has not been well documented. We show here that 5-aminolevulinate synthase mRNA is induced by AIA only in the liver and kidney of rat. Correlating with this, the level of the phenobarbital-inducible cytochrome P-450 b/e mRNAs are elevated by AIA specifically in these tissues.[2] Interestingly, 5-aminolevulinate synthase mRNA in testis is induced by HCG which also induces tissue-specific cytochrome P-450 proteins (24). These results support the proposal (1) that inducibility of 5-aminolevulinate synthase is a secondary consequence of heme depletion due to induction of cytochrome P-450 apoprotein which takes up heme as a prosthetic group.

A basal level of 5-aminolevulinate synthase mRNA was detected in all rat tissues examined. A question which has not been addressed previously is whether heme controls the basal level of 5-aminolevulinate synthase mRNA or only the drug-stimulated increase and also whether this heme control is confined to liver. The results in this paper establish that heme repression of 5-aminolevulinate synthase mRNA levels occurs in all rat tissues studied, with the exception of erythroid spleen, with both basal and induced levels being affected. In erythroid spleen the level of 5-aminolevulinate synthase mRNA was elevated. Possibly this is indirectly due to the induction of heme oxygenase (25). A reservation in this work is that possibly for cell permeability reasons, administered hemin affects only liver 5-aminolevulinate synthase mRNA levels. However, administered 5-aminolevulinate (or its methyl ester in the case of brain) lowers the 5-aminolevulinate synthase mRNA level in all tissues except spleen. It seems most probable that repression by heme is being observed since there is no evidence that 5-aminolevulinate itself has a regulatory role, and it is known that injected 5-aminolevulinate is rapidly converted to heme in many tissues (22).

[2] G. Srivastava, unpublished data.

A final question concerns the level at which heme control is exerted. We have conclusively established that in liver, heme regulates 5-aminolevulinate synthase mRNA levels by acting predominantly, if not exclusively, to inhibit transcription of the 5-aminolevulinate synthase gene.

This work places the 5-aminolevulinate synthase gene in a small group of animal genes (26, 27) known to be negatively controlled by a metabolic end product. The molecular basis for the regulation of the 5-aminolevulinate synthase gene is an important problem to be investigated.

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